INHIBITION OF A COLLAGENASE BY THE HUMAN GINGIVAL MICROBIOTA[1]

JOHN C. THONARD AND HENRY W. SCHERP

Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, New York

Received for publication April 21, 1958

Interactions between the members of a complex indigenous microbiota could be critical determinants of the delicate biochemical balance between the cohabiting microorganisms and the tissues of their host, the status of which determines the relative states of health and disease (Dubos, 1954). The existence of such complex, but little understood, interrelationships in the oral cavity is evident (Bibby *et al.*, 1938; Van Kesteren *et al.*, 1942; Björnesjö, 1950; Thompson and Johnson, 1951). Although numerous antagonisms between members of the oral microbiota have been described, as manifested by inhibition of growth (reviewed by Rosebury *et al.*, 1954), few instances have been reported of the inhibition of a specific biochemical activity of one microorganism by other microorganisms.

In experiments (Thonard and Scherp, 1957) investigating the mechanisms by which human collagen might be lysed during the development of periodontal disease, we incubated untreated gingival tissue with its indigenous microbiota in broth. Collagenolysis could not be demonstrated. In order to show that the gingival tissue contained enough collagen available for enzyme action to be detected by the methods used, control tests were made by adding *Clostridium histolyticum* or filtrates of its broth cultures. Unexpectedly, collagenolysis was undemonstrable in these tests also. However, when *C. histolyticum* filtrates were incubated with gingival tissue in the presence of antibiotics, i. e., when growth of the oral microbiota was suppressed, extensive collagenolysis was demonstrable regularly. Investigation of this anomaly forms the basis for the experiments reported here

MATERIALS AND METHODS

Fresh human gingival tissue from patients suffering from periodontitis simplex[2] was obtained and used as described previously (Thonard and Scherp, 1957). The bovine Achilles tendon used was stored in the frozen state (Schultz-Haudt and Scherp, 1955). A culture of *C. histolyticum* was obtained from Dr. Alworth D. Larson. It was grown and maintained in deep-meat medium and subcultured anaerobically on blood agar or in infusion broth as needed. Filtrates containing collagenase were prepared by growing this organism anaerobically for 3 days in infusion broth at 37 C and filtering through a Selas 02 candle. Approximately 1 g samples of tissues to be sterilized by antibiotics were placed in 10 ml of trypticase-casein-yeast extract (TCY) broth (Schultz-Haudt and Scherp, 1955) containing 200 i.u of penicillin and 200 μg of streptomycin per ml. The tissues were incubated at 37 C overnight, and then washed in 4 or more changes of sterile 0.85 per cent sodium chloride solution. The tissues remained in each change of saline for 1 hr. The tendon was sterilized alternatively by exposure to ethylene oxide vapor as reported by Schultz-Haudt and Scherp (1955).

Samples of tissue were transferred aseptically to tubes containing either 10 ml of TCY broth or 7 ml of TCY broth plus 3 ml of *C. histolyticum* filtrate, as required by the particular experiment (table 1). In experiments requiring exposure of gingival tissue to its indigenous flora (series 1A and 1B), each sample of fresh tissue was divided into 4 or 5 approximately equal portions, 2 of which were placed directly into tubes A and B, respectively; the remaining portions were used as indicated in tubes C and D. In order to expose sterilized tendon to a microbial growth similar to that indigenous to the gingival tissue (series 2A and 2B), cultures of the gingival microbiota were made by shaking pieces of fresh gingival tissue in separate tubes of TCY broth, which were incubated aerobically at 37 C overnight. One-ml portions of the resulting culture were inoculated into the appropriate tubes. Although it is likely that some members of the oral microbiota did not carry over under these circumstances, the proteolytic activity of these cultures

[1] This investigation was aided by a contract, DA-49-007-MD-460, between the Office of the Surgeon General, Department of the Army, and the University of Rochester.

[2] Kindly supplied by Dr. Frederick J. Halik, Periodontist, Rochester, New York.

TABLE 1

Inhibition of Clostridium histolyticum collagenase by oral microbiota

Series	Test	Collagenol
1	A. Gingiva + indigenous flora + *C. histolyticum* filtrate	0/24*
	B. Gingiva + indigenous flora + *C. histolyticum*	0/24
	C. Gingiva (antibiotic sterilized) + *C. histolyticum* filtrate	21/23
	D. Gingiva (antibiotic sterilized) + *C. histolyticum*	9/27
2	A. Tendon (ethox†) + gingival flora	13/18
	B. Tendon (ethox) + gingival flora + *C. histolyticum* filtrate	11/11
	C. Tendon (antibiotic sterilized) + *C. histolyticum* filtrate	16/16
	D. Tendon (antibiotic sterilized) + *C. histolyticum*	13/21
3	A. Gingiva (antibiotic sterilized) + κ-toxin‡	2/2
4§	A. Gingiva + filtrate of series 1A	10/11
	B. Tendon + filtrate of series 1A	2/2
	C. Gingiva + supernatant of series 1A	4/4
	D. Gingiva + filtrate of series 1B	5/6
	E. Gingiva + supernatant of series 1B	4/4
	F. Gingiva + filtrate of gingival starter culture + *C. histolyticum* filtrate	4/6

* Number of tests showing collagenolysis/total number of tests.

† Sterilized by exposure to ethylene oxide vapor (Schultz-Haudt and Scherp, 1955).

‡ Purified collagenase of *Clostridium perfringens* kindly supplied by The Wellcome Research Laboratories, Beckenham, Kent, England.

§ All samples of tissue in this series were sterilized by antibiotics.

on ethylene-oxide-treated bovine tendon accorded with the results reported by Schultz-Haudt and Scherp (1955) and Thonard and Scherp (1957).

All cultures were incubated for 3 days at 37 C aerobically, except those inoculated with *C. histolyticum*, which were incubated anaerobically. The cultures were centrifuged and the supernatant fluids were analyzed for the presence of hydroxyproline-containing peptides as a measure of collagenolysis (Thonard and Scherp, 1957), in comparison with similar cultures not containing the collagenous substrate. In most of the tests recorded as positive, the amounts of hydroxyproline were many times that required as a criterion of minimal collagenolysis.

RESULTS AND DISCUSSION

The results of the tests in series 1A and 1B (table 1) indicate an inhibition of collagenase activity by cultures of the indigenous gingival flora. In the tests of series 1B, the growth of *C. histolyticum* in the presence of the oral microorganisms was confirmed microscopically. Conceivably, the anaerobic sporulating gram-positive bacilli observed might have originated from the gingival flora. This possibility was deemed unlikely, since *Clostridium* species occur very infrequently in cultures from the gingival sulcus (Roth and Myers, 1956). The presence of adequate amounts of collagen in the samples of gingiva and its susceptibility to *C. histolyticum* and *Clostridium perfringens* collagenases were evidenced by the positive results obtained in series 1C, 1D, and 3. Somewhat surprising was the low incidence of collagenolysis in those tests in which antibiotically sterilized tissues were incubated with cultures of *C. histolyticum* (1D and 2D). Microscopical and cultural examination of the negative tests in these two series either failed to disclose any viable bacteria or, in a few instances, revealed long filamentous bizarre forms of bacilli. The latter accord with Gardner's (1940) observation of long filamentous forms of *C. perfringens* under the influence of penicillin in concentrations of the order of one μg per ml. Evidently in our experiments, even repeated saline washes over a period of several hours failed in many cases to elute the antibiotics from the tissues sufficiently to permit normal growth of *C. histolyticum*. Elution from tendon seems to have been more effective. Another factor in these experiments was the possibility that the production of collagenase by *C. histolyticum* was adversely affected by the TCY broth; however, comparison of collagenase production in TCY broth and the infusion broth used for the *C. histolyticum* filtrates failed to reveal any differences.

On first examination, the results of the tests with tendon sterilized by ethylene oxide (series 2A and 2B) seem to be inconsistent with those

of the tests with gingiva. Previous studies (Schultz-Haudt and Scherp, 1955; Thonard and Scherp, 1957) have shown that exposure of collagen to ethylene oxide renders it susceptible to digestion by 70 per cent of cultures of the gingival flora from cases of periodontitis simplex. The results of series 2A confirm this observation. If the gingival flora inhibited the collagenase of *C. histolyticum*, collagenolysis should have occurred at the same rate in series 2B, whereas in fact it occurred in all tests. This discrepancy could be explained by the fact that *C. histolyticum* produces not only a specific collagenase but also two other proteases (MacLennan *et al.*, 1958), of which only the collagenase seems to be inhibited by the oral microbiota. The increased incidence of collagenolysis in series 2B would accordingly be attributable to one or both of the nonspecific proteases acting in conjunction with oral microbial enzymes upon the collagenous substrate, which had been rendered more digestible by prior treatment with ethylene oxide.

To account for the complete lack of collagenolysis in series 1A and 1B, several explanations seemed possible: (a) the oral bacteria could be utilizing the collagenase metabolically or simply destroying it, (b) collagenase may not even have been produced by *C. histolyticum* in the presence of the oral bacteria, and (c) products of the oral bacteria could be inhibiting the collagenase. Additional experiments were undertaken to evaluate these possibilities (series 4, table 1).

After removal of samples to test for the presence of hydroxyproline, the remaining broth of the tests in series 1A was either filtered through Selas 02 filters or centrifuged at 3000 rpm for 20 min and then retested for collagenase activity against fresh gingival tissue or bovine tendon in TCY broth containing 200 i.u. of penicillin and 200 μg of streptomycin per ml. Collagenolysis occurred in 16 of the 17 retests (4A, B, C). Clearly, the oral bacterial cultures had not destroyed the collagenase.

Similar experiments with the remaining broth of the tests in series 1B showed collagenolysis in 9 out of 10 retests (4D, E). Evidently, *C. histolyticum* had produced collagenase while growing in the presence of the oral bacteria.

These results indicated that the oral microbiota effects a reversible inhibition of *C. histolyticum* collagenase. In order to test for the production of a soluble collagenase inhibitor, starter cultures in TCY broth were made as described previously, incubated aerobically for 48 hr at 37 C, and filtered through Selas 02 filters. A 7 ml portion of each filtrate was incubated for 3 days at 37 C with its homologous gingival tissue, which had been stored during the interim in saline at 4 C, plus 3 ml of filtrate from *C. histolyticum*. Sterility was maintained by a final concentration of 200 i.u. of penicillin and 200 μg of streptomycin per ml. Collagenolysis was obtained in 4 of 6 tests (4F). These results indicated that a soluble collagenase inhibitor is at best irregularly present in cultures of the gingival bacteria and that the mechanism of inhibition is intimately associated with the presence of the bacterial cells themselves.

The present observations recall the investigation of the antibacterial action of human saliva by Bibby *et al.* (1938), who reported: "The antibacterial agent effective against *Micrococcus lysodeikticus* is unable to pass a Berkefeld filter, is more closely associated with the salivary bacteria and cells and gives more definite evidence of its elaboration by bacterial cultures."

Although several instances of the inhibition of a specific activity of one organism by antagonistic effects of another organism have been recorded, we still have little knowledge as to how such effects are mediated. Gaté and Papacostas (1921) noticed that the symptoms of infection by *Corynebacterium diphtheriae* were much milder if Friedländer's bacillus was associated with the infection. However, with intramuscular injection in guinea pig thighs of mixed filtrates of these two organisms grown separately, no interference with the lethal effect of the diphtherial toxin was observed. On the other hand, when *C. diphtheriae* was grown in broth with Friedländer's bacillus or its culture filtrates, toxin production was decreased, presumably because growth of the *C. diphtheriae* was poor also. Alternatively, the inhibition of toxin production might have resulted from a disturbance of the iron metabolism of *C. diphtheriae*, which is now known to be a critical determinant of its toxin production (Pappenheimer, 1947). Hall and Peterson (1923) found that toxin production by *Clostridium botulinum* was markedly inhibited when it was grown in glucose broth with various aerobic acidogenic soil bacteria. The latter bacteria were shown also to destroy preformed botulinus toxin. Both the inhibition of toxin formation and the destruction of toxin were due not to the acidity as such but to undefined factors effective under acid conditions. Ramon *et al.* (1945) reported that filtrates of *Bacillus*

subtilis cultures inhibited the growth *in vitro* of a number of microorganisms, including staphylococci, *Bacillus anthracis*, and *C. diphtheriae*, and inhibited *in vivo* the action of their toxins. Similar filtrates inactivated the viruses of rabies and equine encephalomyelitis (Remlinger and Bailly, 1946*a*, *b*) probably due to unidentified proteases. Thompson and Johnson (1951) were able to show that the inhibition of growth of *C. diphtheriae* by *Streptococcus mitis* and other oral viridans streptococci correlated with the amount of hydrogen peroxide produced by the streptococci and that this inhibition could be reversed by catalase-producing staphylococci.

Elucidation of the mechanisms of antagonisms like those discovered in the present investigation, and determination of the significance of analogous reactions in the ecology of the oral flora will require extensive additional experimentation.

SUMMARY

When fresh human gingival tissue from patients suffering from periodontitis simplex was incubated for 3 days at 37 C in broth with its indigenous flora, plus either growing cells or culture filtrates of *Clostridium histolyticum* as sources of collagenase, no collagenolysis was detectable chemically. When the growth of the gingival microbiota was suppressed by antibiotics, the inhibition of collagenase was averted. The growth of the gingival microbiota did not destroy preformed collagenase or suppress its formation by *C. histolyticum*; rather, it effected a reversible inhibition of this enzyme. The formation of a soluble inhibitor of collagenase in cultures of the gingival bacteria could not be demonstrated; the inhibitory mechanisms required the continued presence of the bacterial cells. Altered collagen, in the form of bovine Achilles tendon sterilized by ethylene oxide, was lysed by 70 per cent of cultures of the gingival biota alone, and by all such cultures (presumably by proteases other than collagenase) when *C. histolyticum* filtrate was added.

REFERENCES

BIBBY, B. G., HINE, M. K., AND CLOUGH, O. W. 1938 The antibacterial action of human saliva. J. Am. Dental Assoc., **25**, 1290–1302.

BJÖRNESJÖ, K. B. 1950 Studies on the antibacterial factors of human saliva. Acta Chem. Scand., **4**, 835–845.

DUBOS, R. J. 1954 In *Biochemical determinants of microbial diseases*. Harvard University Press, Cambridge, Massachusetts.

GARDNER, A. D. 1940 Morphological effects of penicillin on bacteria. Nature, **146**, 837–838.

GATÉ, J. AND PAPACOSTAS, G. 1921 Antagonisme biologique entre le Bacille de Löffler et le Pneumobacille de Friedlander. Compt. rend. soc. biol., **85**, 859–861.

HALL, I. C. AND PETERSON, E. 1923 The effect of certain bacteria upon the toxin production of *Bacillus botulinus in vitro*. J. Bacteriol., **8**, 319–341.

MACLENNAN, J. D., MANDL, I., AND HOWES, E. L. 1958 New proteolytic enzymes from *Clostridium histolyticum* filtrates. J. Gen. Microbiol., **18**, 1–8.

PAPPENHEIMER, A. M., JR. 1947 Diphtheria toxin. III. A reinvestigation of the effect of iron on toxin and porphyrin production. J. Biol. Chem., **167**, 251–259.

RAMON, G., RICHOU, R., AND RAMON, P. 1945 Culture du *B. subtilis* en milieu de composition chimique définie, en vue de la production et de l'extraction du principe antagoniste des bactéries pathogènes et des toxines microbiennes. Compt. rend., **221**, 165–167.

REMLINGER, P. AND BAILLY, J. 1946*a* Action des filtrats de cultures de *Bacillus subtilis* sur le virus de la rage. Compt. rend., **223**, 118–120.

REMLINGER, P. AND BAILLY, J. 1946*b* Action du filtrat de cultures de *Bacillus subtilis* sur le virus de l'Encéphalomyélite des équidés. Compt. rend., **223**, 267–268.

ROSEBURY, T., GALE, G., AND TAYLOR, D. F. 1954 An approach to the study of interactive phenomena among microorganisms indigenous to man. J. Bacteriol., **67**, 135–152.

ROTH, G. D. AND MYERS, H. I. 1956 Hide powder and collagen lysis by organisms from the oral cavity. Oral Surg. Oral Med. Oral Pathol., **9**, 1172–1184.

SCHULTZ-HAUDT, S. D. AND SCHERP, H. W. 1955 Lysis of collagen by human gingival bacteria. Proc. Soc. Exptl. Biol. Med., **89**, 697–700.

THOMPSON, R. AND JOHNSON, A. 1951 The inhibitory action of saliva on the diphtheria bacillus: hydrogen peroxide, the inhibitory agent produced by salivary streptococci. J. Infectious Diseases, **88**, 81–85.

THONARD, J. C. AND SCHERP, H. W. 1957 Resistance of human gingival collagen to human gingival bacteria. Proc. Soc. Exptl. Biol. Med., **96**, 165–170.

VAN KESTEREN, M., BIBBY, B. G., AND BERRY, G. P. 1942 Studies on the antibacterial factors of human saliva. J. Bacteriol., **43**, 573–583.